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AUTHORITY

U.S. Army Medical Research and Materiel Command ltr., dtd August 9, 2001.

AD__________________

GRANT NUMBER DAMD17-97-1-7069

TITLE: Differential Regulation of Cell Cycle Progression in Human Breast Cancer

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REPORT DATE: August 1998

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 1998	Annual (1 Aug 97 - 31 Jul 98)

4. TITLE AND SUBTITLE

Differential Regulation of Cell Cycle Progression in Human Breast Cancer

5. FUNDING NUMBERS

DAMD17-97-1-7069

6. AUTHOR(S)

Direnzo, James, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Dana Farber Cancer Institute
Boston, Massachusetts 02115-6084

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

19990108 041

11. SUPPLEMENTARY NOTES

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Factors that mediate chemical or structural alterations of chromatin have been implicated as mediators of nuclear receptor function. Prominent in this diverse group of putative coactivators is the Steroid Receptor Coactivator-1 (SRC-1) and its related factors. These factors physically interact with liganded nuclear receptors and couple them to the multifunctional coactivators such as p300 and CPB which have both intrinsic and associated histone acetyltransferase (HAT) activity. Other studies have suggested that factors involved in the structural remodeling of chromatin are required for nuclear receptor function. One such factor, the Brahma Related Gene-1 (BRG-1) potentiates nuclear receptor signaling and has been shown to be associated with liganded nuclear receptors. Like p300 and CBP, we find that BRG-1 exists in a stable complex with SRC-1, and this complex can be recruited to a hormone bound estrogen receptor (ER). We demonstrate that *in vitro* the hormone-dependent association between BRG-1 and ER is mediated by SRC-1, and that structural determinants of ER required for SRC-1 binding overlap with those required for the association between ER and BRG-1. Furthermore, we present data that coactivation of ER signaling by either SRC-1 or CBP requires BRG-1. In addition we show that the BRG-1-mediated coactivation of ER signaling is synergistically enhanced by inhibition of histone deactylation. These studies suggest that SRC-1 functions as an adaptor that links two distinct classes of coactivators to nuclear receptor signaling.

14. SUBJECT TERMS
Breast Cancer
Estrogen Receptor

15. NUMBER OF PAGES
39

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Limited

FOREWORD

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8/4/98

PI - Signature   Date

July 28, 1998

To: Research Data Management

Fr: James DiRenzo Ph.D.

Re: Annual Report for Grant Number DAMD17-97-1-7069

Sirs:

Enclosed is a description of the progress made in the past 12 months which was funded by the garnt number listed above. Topics will be adressed in the order in which they appear on the Statement of Work, which can be found on page III-B.2.e of the original proposal. Also enclosed as Appendix 1 is a manuscript that is to submitted for publication by 7/30/98. This manuscript most directly addresses topic IV of the Statement of Work as well as other topics.

Thank You very Much

7/28/98

James DiRenzo Ph. D.

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Research Progress for Grant number DAMD17-97-1-7069

Introduction

Background

Breast cancer is the most common cancer afflicting women in the United States and the second leading cause of all cancer deaths. Enhanced understanding of the molecular mechanisms involved in the initiation and progression of breast cancer will likely lead to better methods for prevention, detection and treatment. Critical predictions as to the biological behavior, and thus the appropriate therapeutic strategy, of breast cancers can be made based upon the status of the estrogen receptor (ER). Approximately 50% of all breast cancers are characterized by elevated levels of ER and, of these, approximately 2/3 exhibit estrogen-dependent growth, implying a positive role for ER in cell cycle progression. In addition to these, breast cancers have been characterized in which ER is undetectable, and these are refractory to antiestrogen therapy and have greater metastatic tendencies. Several studies have suggested that the repression of the gene encoding ER is required for these cells to proliferate suggesting that ER may also play a role in the negative regulation of the cell cycle (1, 2). Taken together, these studies suggest that ER is capable of mediating both positive and negative effects upon the growth of breast cancer cell lines. A greater understanding of these diverse effects will likely lead to the identification of new potential targets of pharmacological intervention against breast cancer.

Several useful breast cancer cell lines have been characterized, that provide a model of the biological features of both ER-positive and ER-negative breast cancers. Studies using the human ER-positive breast cancer cell line MCF-7 have demonstrated that these cells are depenedent upon the presence of estrogen to continue to progress through the cell cycle (3). Treatment of these cells with antiestrogens has been shown to cause a block in the first gap phase (G1), which is characterized by an early reduction in expression of cyclin D1 a critical regulator of the progression from G1 to S in the cell cycle (4). This early negative effect on cell cycle progression from G1 to S appears to be compounded by a subsequent increase in expression of two potent inhibitors of cyclin-dependent kinase (CDK) activity, $p27^{KIP1}$, and $p21^{CIP1}$ (3). These studies provide strong evidence that disruption of the estrogen signalling pathway causes a block in G1 in ER-positive breast cancer cells, and suggest that this block may be the result of both positive and negative regulation of genes encoding critical cell cycle regulators. In contrast to MCF-7 cells, the human breast cancer cell line MDA-MB-231 is ER-negative, and thus refractory to antiestrogen treatment (5). The sharp decrease in ER expression that has been correlated with reduced therapeutic potential of antiestrogen treatment lead to the hypothesis that ectopic expression of ER in ER-negative cells breast cancer cells would reconstitute estrogen-dependent growth, and thus sensitivity to antiestrogens. In these experiments, exogenous ER failed to revert ER-negative cell lines to a state of sensitivity to antiestrogens, and had the somewhat paradoxical effect of making the continued growth of these cells sensitive to estrogen treatment (2, 5). These studies, coupled with the growth promoting effects of estrogen in ER-positive cells suggest that ER is capable of exerting differential effects on the cell cycle.

The estrogen receptor is a member of the nuclear receptor superfamily of ligand activated transcription factors that regulate growth, differentiation, and homeostasis in eukaryotic cells [Evans, 1988 #398]. They exert their functions through the regulation of specific target genes in response to their cognate hormones, as well as other transduced signals. The estrogen response element (ERE) is highly conserved, and is absolutely required for the direct regulation of gene expression by estrogens (6). It is composed of a palindromic arrangement of two ERE "half-sites" spaced by three random base pairs. The estrogen receptor binds to its response element in the regulatory region of target genes as a homodimer, and is believed to activate transcription through a hormone-dependent association with a large complex of proteins that integrate and transmit transactivating signals to the basal transcription machinery (7-11). This interaction is thought to be mediated through a well conserved region of the hormone binding domain (HBD), known as the activating function region-2 (AF-2) (12). Structural studies of the HBD of other nuclear receptors have shown that the AF-2 region forms an α-helix, and that the position of this helix relative to the strucural ligand binding pocket is alterred as a result of ligand binding (13, 14). This conformational change is believed to be required for the interaction between nuclear receptors and their coactivators, a step which is essential for the transmission of the hormone binding signal to the basal transcription machinery.

Critical to the understanding of the mechanisms of action of the nuclear receptors is the identification of factors that mediate the hormone binding signals between the receptor and the basal transcriptional machinery. Studies of the structural domains of nuclear receptors suggest that there are at least two regions of the receptor which contain transactivation functions. The activating function region 1 (AF-1) is located in the poorly conserved N-terminus of some nuclear receptors, and is capable of transactivating in the absence of ligand (15). Recently, this region was demonstrated to be a target of the MAP-kinase regulatory pathway, suggesting a convergence of steroidal signalling, and peptide growth factor signalling (16). Ligand-dependent transactivation has been mapped to the C-terminal region of the HBD of several nuclear receptors. The demonstration of the requirement of the AF-2 for ligand-dependent transactivation (12) has lead to a concerted effort to identify and characterize proteins which interact with the AF-2 region in a ligand-dependent manner. Such proteins are thought to be excellent candidates for ligand-dependent coactivators of nuclear receptor signalling. Several proteins have been identified which interact with the AF-2 domain of one or more nuclear receptors, and do so in a manner that is ligand-dependent. Direct protein/protein interaction screening, has lead to the detection and identification of several proteins which interact with ER in a manner that is dependent upon the presence of estrogen (8, 9, 17). These proteins, ERAP 140, RIP 140, SRC-1, TIF-2 and AIB-1

have all been demonstrated to interact with the AF-2 of one or more nuclear receptors in the presence of ligand (8, 9, 17). Moreover, SRC-1 has also been demonstrated to potentiate a transcriptional response by several steroid receptors. Comparisons of the primary amino acid sequences of these three proteins have revealed no significant homology with each other, suggesting that more than one biochemical pathway may be utilized to mediate transcriptional activation from nuclear receptors. This idea is consistent with the notion that nuclear receptors may differentially regulate target genes via interactions with a variety of coactivators present.

I. Mutagenesis of human estrogen receptor (ER)

Studies of the role of the estrogen receptor (ER) in the promotion and progression of breast cancer began with the generation of mutations in the cDNA encoding ER that would allow us to determine the relative contributions of the two activating functions of ER, AF-1 and AF-2 to two important aspects of estrogen function in breast cancer, regulation of estrogen responsive genes and promotion of cell cycle progression. Mutations were developed that deleted either the N-Terminal AF-1, the C-Terminal AF-2 or both. These mutants were subcloned into a variety of expression plasmids that would be useful for production of recombinant protein in bacteria, transient expression in mammalian cells, and stable expression in mammalian cells. All subclones were rigorously checked for accuracy and efficacy.

II. Identification of domains of ER which are critical to hormone dependent growth suppression in MDA-MB-231.

The human breast cancer cell line MDA-MB-231 is an ER-negative cell line that had been previously demonstrated to be growth inhibited by the actions of of stably overexpressed ER. In our studies, stable cell lines were developed that overexpressed wild type and mutated versions of ER in MDA-MB-231. Each cell line was selected for resistance to neomycin, and then screened for expression of the encoded version of ER. During the selection of these clonal cell lines, it was observed that cells overexpressing wt ER and ER lacking AF-1 grew slowly and eventually growth arrested. Those cell lines which did grow were observed to express only low amounts of either wt ER or ER lacking the AF-1. In contrast, cell lines which expressed ER lacking the AF-2 grew more vigorously and several expressed high levels of the encoded mutant. Use of anti-estrogens provided some relief to this slow-growth phenotype. Based upon these observations colony forming assays were carried out and it was determined that while wt ER and the ER lacking AF-1 grew slowly in the presence and absence of anti-estrogens, the ER-lacking AF-2 mutant did not effect growth and was not capable of mediating a growth arrest in response to either estrogen or anti-estrogens.

The finding that these cell lines were sensitive to wt ER and ER lacking the AF-1 but not ER lacking the AF-2 suggested that the growth inhibitory effects are likely to be mediated through the AF-2 region of the receptor. However the fact that cell lines that express moderate to high levels of wt ER were not viable has made further progress on this aspect of the proposal difficult. What could be determined was that the AF-2 region of ER which is known to be required for hormone-dependent regulation of target genes appears to have a role in the hormone-dependent

growth suppression of MDA-MB-231 cells. This information has been further explored at the biochemical level (see Sec IV).

III. Identification of ER mutants capable of exerting a dominant negative effect over estrogen-dependent growth in MCF-7.

Similar studies using the estrogen-dependent breast cancer cell line have been undertaken to determine if the AF-2 region of ER is responsible for mediating the positive effects cell cycle progression observed in these cell. In these studies wt and mutant ER were over expressed in MCF-7 cells and assayed first for their ability to modulate the endogenous estrogen response observed in transient transfection assays. In these assays, it was observed that the ER mutant lacking the AF-1 had the effect of reducing the overall rate of transcription from an estrogen responsive reporter, but had only subtle effects on the relative induction in response to hormone. This result is consistent with the previous reports that the AF-1 region contributes to gene activation in a hormone-independent manner. The mutant ER lacking the AF-2 was observed to have a dominant negative effect over the endogenous estrogen response observed in MCF-7 cells. Futre studies in this area will focus on methods of transiently transfectin this and other ER mutants in MCF-7 cells and measuring the effects on cell cycle progression.

VI. Characterization/idenbtification of ER-associatedproteins which interact with domains of ER which are critical to cell cycle regulation.

The majority of the progress to report in this annual report is most directly applicable to section IV. Attached as Appendix 1 is work has recently been submitted for publication and and the author respectfully requests that this information be treated as unpublished material until such time that it has been accepted. This work describes a novel protein complex that has the ability to interact with the estrogen receptor in the presence of estrogen and also contains factors that are known to play an important role in the structural remodeling of the chromatin in which all genes are housed. Chromatin remodeling is an important global regulator of gene activation. We believe that this work provides a novel insight into the mechanism by which estrogen signaling can be physically coupled to chromatin remodeling. Please see Appendix one which is a complete copy of the manuscript which has been submitted to the journal *Molecular Cell.*

Studies ongoing in this area are aimed at a continued characterization of this protein complex. Efforts are currently underway to identify other proteins which co-purify with the complex, and preparations are being made for a larger scale purification stategy that will allow us to determine if this complex has chromatin remodeling properties in vitro.

The author respectfully refers the review committee to Appendix 1.

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Recruitment of BRG-1 by SRC-1 is Required for Estrogen Receptor Activation: Convergence of Coactivator Pathways in Steroid Receptor Signaling

Running Title: ER Coactivator Convergence

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Abstract

Factors that mediate chemical or structural alterations of chromatin have been implicated as mediators of nuclear receptor function. Prominent in this diverse group of putative coactivators is the Steroid Receptor Coactivator-1 (SRC-1) and its related factors. These factors physically interact with liganded nuclear receptors and couple them to the multifunctional coactivators such as p300 and CPB which have both intrinsic and associated histone acetyltransferase (HAT) activity. Other studies have suggested that factors involved in the structural remodeling of chromatin are required for nuclear receptor function. One such factor, the Brahma Related Gene-1 (BRG-1) potentiates nuclear receptor signaling and has been shown to be associated with liganded nuclear receptors. Like p300 and CBP, we find that BRG-1 exists in a stable complex with SRC-1, and this complex can be recruited to a hormone bound estrogen receptor (ER). We demonstrate that *in vitro* the hormone-dependent association between BRG-1 and ER is mediated by SRC-1, and that structural determinants of ER required for SRC-1 binding overlap with those required for the association between ER and BRG-1. Furthermore, we present data that coactivation of ER signaling by either SRC-1 or CBP requires BRG-1. In addition we show that the BRG-1-mediated coactivation of ER signaling is synergistically enhanced by inhibition of histone deactylation. These studies suggest that SRC-1 functions as an adaptor that links two distinct classes of coactivators to nuclear receptor signaling.

The estrogen receptor (ER) is a member of a large family of ligand activated transcription factors that control a cells ability to divide, to acquire and execute physiologic functions and ultimately to program cell death. These factors exert both positive and negative control over target genes by binding to sequence specific response elements located in the regulatory regions of such genes. Due to the ability of ligand to regulate their activity, these factors have proven to be a useful system in which to study the mechanisms by which this positive and negative transcriptional regulation occurs (Glass et al., 1997; Horwitz et al., 1996). In the absence of hormone, many receptors actively repress transcription via direct interactions with corepressors such as NCoR SMRT and SunCoR (Chen and Evans, 1995; Horlein et al., 1995; Zamir et al., 1997). Upon hormone binding, these corepressor complexes dissociate and liganded receptors interact with distinct multiprotein complexes that function to transmit activating signals to the general transcription machinery (Cavaillès et al., 1994; Halachmi et al., 1994; Kurokawa et al., 1995). The mechanisms by which a hormone binding signal is transmitted are not clearly understood, however studies suggest that some aspects of general transcription factor (GTF) function may be targeted directly (Abraham et al., 1993). Other studies suggest that these coactivator complexes may influence structure and chemical composition of chromatin thereby modulating GTF assembly and activity (Kamei et al., 1996; Kraus and Kadonaga, 1998; Ogryzko et al., 1996; Spencer et al., 1997)

A variety of putative coactivators have been identified based primarily upon their ability to interact with a nuclear receptor in a hormone-dependent manner. Prominent in this diverse group is the Steroid Receptor Coactivator-1 (SRC-1/NCoA-1) (Onate et al., 1995; Torchia et al., 1997) and its related factors TIF2/GRIP-1/NCoA-2 (Hong et al., 1997; Torchia et al., 1997; Voegel et al., 1996) and RAC-3/AIB1/PCIP/ACTR/TRAM (Anzick et al., 1997; Chen et al., 1997p; Li et al., 1997; Takeshita et al., 1997; Torchia et al., 1997). These factors physically interact with many members of the receptor superfamily, and have been shown in functional assays to enhance hormone-dependent transcriptional activation by nuclear receptors (Anzick et al., 1997; Chen et al., 1997; Li et al., 1997; Onate et al., 1995; Takeshita et al., 1997; Torchia et al., 1997; Voegel et al., 1996). An important insight into one mechanism by which the SRC-1 family potentiates nuclear receptor signaling was gained by the demonstration of a stable interaction between SRC-1 and the

CREB-Binding Protein (CBP) as well as its homolog p300 (Hanstein et al., 1996; Kamei et al., 1996; Yao et al., 1996). These multifunctional transcriptional coactivators have been proposed to effect gene activation through both direct interactions with the RNA polymerase II complex (Abraham et al., 1993) and also via intrinsic and associated histone acetyltransferase (HAT) activities (Bannister and Kouzarides, 1996; Chen et al., 1997; Scolnick et al., 1997; Spencer et al., 1997). These studies suggest that this complex may mediate nuclear receptor activation is through the acetylation of histones and possibly other targets. These findings are consistent with a body of evidence that decompaction of chromatin via either chemical or structural modification correlates with the activation of genes (Paranjape et al., 1994). They also provide a biochemical link between nuclear receptor signaling and modulation of chromatin structure.

As the primary unit of chromatin, the nucleosome has been shown to be the target of both chemical and structural modification. Several studies have shown that the acetylation of specific lysine resdiues within core histones results in a reduced affintiy for DNA, making acetylated chromatin more accesible to transcriptional regulators (Imbalzano et al., 1994; Ostlund Farrants et al., 1997). These effects are complemented by structural modifications of chromatin which include both disruption and relocation of nucleosomes (Cairns, 1998). Interestingly, recent reports have shown that mutations in a yeast HAT complex were phenotypically similar to mutations in components of the nucleosome remodeling Swi/Snf complex and likewise, were rescued by suppressor mutations in several components of chromatin (Pollard and Peterson, 1997). Several distinct multiprotein complexes have been identified purified and shown to possess an intrinsic ability to remodel chromatin (Cairns et al., 1996; Ito et al., 1997; Kwon et al., 1994; Peterson et al., 1994; Tsukiyama et al., 1995; Varga-Weisz et al., 1997). Each of these complexes does so in a manner that is strictly ATP-dependent and, each contains a member of the Swi-2/Snf-2 family of nuclear ATPases. The human homologs of ySwi-2, hBrm and hBRG-1 (hSnf-2α and hSnf-2β respectively) (Chiba et al., 1994) are crucial to the function of the human Swi/Snf nucleosome remodeling complex (Kwon et al., 1994). Brm and BRG-1 have been shown to interact with various nuclear receptors in a yeast-based two-hybrid assay (Ichinose et al., 1997) and also have been shown by transient transfection to be essential for nuclear receptor signaling (Chiba et al.,

1994). Other studies have demonstrated that BRG-1 will form a complex with the retinoblastoma gene product (Dunaief et al., 1994) and that this complex is capable of cooperatively coactivating glucocorticoid receptor (GR) signaling (Singh et al., 1995). More recent studies have shown that a complex containing two components of the hSwi/Snf complex, BRG-1 and BAF-155 (Swi-3), is required for GR-mediated chromatin remodeling and transcriptional activation of a stably integrated reporter (Fryer and Archer, 1998). Taken together, these studies suggest that BRG-1 plays an important role in the activation of transcription and chromatin remodeling (Fryer and Archer, 1998) that follows nuclear receptor activation.

In order to better understand the mechanisms of transcriptional activation by nuclear receptors we sought to identify interacting factors that were specifically co-immunoprecipitated with monoclonal antibodies directed against human SRC-1. We demonstrate that, like CBP and p300, BRG-1 exists in a complex with SRC-1 in whole cell lysates. We show that this complex can be recruited to an activated estrogen receptor (ER), in a manner that is both hormone and AF-2 dependent. *In vitro* studies demonstrate that the hormone dependent association between BRG-1 and ER requires SRC-1. Furthermore, we demonstrate that BRG-1 is required for hormone-dependent transcriptional activation by ER and that coactivation by either SRC-1 or CBP requires BRG-1 function. Finally we demonstrate that the BRG-1 mediated coactivation of ER signalling acts synergistically with Trichostatin A (Minucci et al., 1997) a potent inhibitor of histone deacetylation suggesting that the coactivator function of BRG-1 is regulated by mechanisms that modulate the acetylation state of chromatin.

Results

Given the previous finding that SRC-1 directly associates with ER in response to agonist (Halachmi et al., 1994; Hanstein et al., 1996) monoclonal antibodies were raised against SRC-1, and used to identify associated endogenous cellular factors by co-immunoprecipitation with SRC-1. Each of the antibodies characterized was capable of immunoprecipitating the 160 kDa SRC-1 from whole cell extracts (WCE) of metabolically labelled MDA-MB-231 cells, and other cell lines (Fig. 1A shaded arrow). One monoclonal antibody, GT-16, was unique, in that it consistently co-

precipitated a ~200 kDa protein (Fig. 1A unshaded arrow) from a variety of cell lines. This factor was not recognized by any of the α-SRC-1 monoclonals in western blot analysis, indicating that it was not an SRC-1 family member or homologue. Since previous studies had shown that BRG-1 and Brm were critical for receptor function (Chiba et al., 1994; Muchardt and Yaniv, 1993), we sought to determine if the 200 kDa protein co-precipitated by GT-16 was either BRG-1 or Brm. Western blotting of α-SRC-1 immunoprecipitates with a polyclonal serum directed against hBRG-1, show that BRG-1 is specifically coprecipitated by GT-16 and not by other α-SRC-1 monoclonal antibodies tested (Fig. 1B). Experiments using other cell lines show that the structural homolog of BRG-1, hBrm is similarly co-precipitated by GT-16 (data not shown). It is noteworthy that GT-16 failed to directly immunoprecipitate in vitro translated ^{35}S-labelled BRG-1, demonstrating that BRG-1 was co-immunoprecipitated with SRC-1, and not directly immunoprecipitated as a result of cross-reactivity between GT16 and BRG-1 (data not shown). To confirm the identity of the 200 kDa protein as BRG-1, α-SRC-1 immunoprecipitates from metabolically labelled MCF-7 cells were boiled in detergent and reprecipitated with antibodies directed against SRC-1 and BRG-1. In these studies, reprecipitation of BRG-1 was observed from αBRG-1 and GT-16 primary immunoprecipitates, and not from GT-12 or a non-specific antibody (Fig. 1C). These experiments provide direct evidence that the ~200 kDa factor coprecipitated by GT-16 in Figure 1A is BRG-1. Taken together these studies clearly demonstrate that SRC-1 and BRG-1 exist in a stable complex present in whole cell extracts.

The interaction between SRC-1 and BRG-1 suggested that SRC-1 may mediate the association between nuclear receptors and BRG-1, that depends on the presence of hormone and an intact hormone dependent activation domain (AF-2). To test this a glutathione-S-transferase/Estrogen Receptor hormone binding domain (GST-HBD) fusion protein, and a corresponding AF-2 deletion mutant (GST-Δ534), chosen because it retains hormone binding, but fails to interact with SRC-1 were immobilized on glutathione-linked agarose. These fusions were

used as affinity matrices to enrich for interacting proteins from metabolically labelled MCF-7 cells, in the absence or presence of estrogen. Following this affinity step, retained fractions were boiled in detergent and subjected to reprecipitation with antibodies directed against either non-specific controls (not shown), SRC-1 or BRG-1 (Fig. 2A). Antibodies directed against SRC-1 and BRG-1 efficiently reprecipitated their respective antigen targets exclusively from fractions enriched by estrogen bound GST-HBD. This enrichment was strictly dependent upon the presence of an agonist and the AF-2 domain. These experiments demonstrate an estrogen-dependent association betwen ER and BRG-1 and suggest that the structural requirements for this association overlap with those required for the interaction between SRC-1 and ER.

In order to determine if the association between ER and BRG-1 was mediated by SRC-1, similar GST-pulldown experiments were carried out using in vitro translated radiolabelled BRG-1 and in vitro translated unlabelled SRC-1 (Figure 2B). In the absence of exogenous SRC-1, BRG-1 interacted with ER in the absence and presence of ligand, which was in contrast to the agonist dependence observed in whole cell extracts. The addition of SRC-1 to these reactions restored an estrogen dependent interaction between BRG-1 and ER that was similar to that observed in experiments using cellular extracts. These studies imply that the interaction between ER and BRG-1 seen in the absence of SRC-1 may be non-physiologic and that the physiologic association between ER and BRG-1 requires SRC-1. These data, coupled to the observations of a stable interaction between SRC-1 and BRG-1 support the conclusion that SRC-1 mediates the hormone dependent interaction between BRG-1 and ER.

The observation that the HBD of ER was capable of associating with the SRC-1/BRG-1 complex in the presence of estrogen, implied that the SRC-1/BRG-1 complex may function as a coactivator of ER-mediated transcriptional activation. To determine if BRG-1 could potentiate ER-mediated signaling, transient transfections were carried out in the BRG-1/Brm-deficient adrenal carcinoma cell line, SW13. In these cells it was observed that, even in the presence of overexpressed ER, activation of an estrogen responsive reporter by estradiol was strictly dependent upon overexpression of BRG-1 (Fig. 3A). This transcriptional response requires an intact AF-2 domain, confirming that structural requirements for BRG-1 mediated coactivation also overlap with those required for the interaction between SRC-1 and ER. The observed requirement for BRG-1

coupled to western blot analysis indicating expression of SRC-1 in (data not shown) suggest that SRC-1 is not sufficient to mediate transcriptional activation by ER. In similar studies, little or no coactivation of estrogen signalling was observed by overexpression of either SRC-1 (Figure 3B) or its family member RAC3 (Li et al., 1997) (data not shown), and the combination of BRG-1 and SRC-1 resulted in a coactivation that exceeded either factor alone (Fig.3B). Taken together, these studies demonstrate that BRG-1 is required for ER-mediated transcriptional activation, and that the coactivator function of SRC-1 family members likewise depends on BRG-1 activity.

Recent studies have shown that SRC-1 and its family members interact with potent histone acetyltransferases (Chen et al., 1997; Hanstein et al., 1996; Kamei et al., 1996; Spencer et al., 1997; Yang et al., 1996; Yao et al., 1996) and may also contain weak HAT activity (Chen et al., 1997; Spencer et al., 1997). In light of these studies and our data demonstrating a biochemical interaction between SRC-1 and BRG-1, we sought to determine if BRG-1 could modulate the ability of either SRC-1 or CBP to coactivate nuclear receptor signalling. As shown in Figure 3B, overexpression of SRC-1 augmented BRG-1 mediated coactivation of ER but was insufficient to coactivate ER in the absence of BRG-1. Similarly, overexpression of CBP was observed to have little effect on ER signaling in the absence of BRG-1 but was capable of enhancing BRG-1 mediated coactivation (Fig. 4A). One possibility that would account for the requirement of BRG-1 by both SRC-1 and CBP is that BRG-1 may exert its function cooperatively with histone acetylation. To test this idea, the histone deacetylase inhibitor Trichostatin A (TSA) (Minucci et al., 1997) was tested for its ability to modulate the BRG-1-dependent coactivation of ER signalling by BRG-1. In the absence of exogenous BRG-1 neither estrogen nor TSA could stimulate ER-mediated transcription, however, together they elicited a seven fold induction relative to untreated controls (Fig. 4B) suggesting that one mechanism by which ER function is repressed in SW-13 cells involves the modulation of histone acetylation. Additionally, the reintroduction into SW-13 cells of BRG-1, but not the ATPase deficient BRG-1 mutant, K798R (Khavari et al., 1993), elicited a synergistic activation of ER signaling in response to estrogen and Trichostatin A (Figure 4B). Taken together, these experiments suggest that the ability of BRG-1 to coactivate ER signaling may be enhanced via increased acetylation of histones. Furthermore, they also suggest that BRG-1 is required in order for histone acetylation to fully coactivate ER. Finally, these

studies show that coactivation of ER by BRG-1 is dependent upon an intact BRG-1 ATPase domain, consistent with other studies in which the function of BRG-1 is ATP-dependent (Kwon et al., 1994). Taken together, these results demonstrate that factors involved in two distinct mechanisms of transcriptional coactivation, histone acetylation and chromatin remodeling, are functionally linked through the physical association between SRC-1 and BRG-1.

Discussion

Both histone acetylation and BRG-1-dependent chromatin remodeling have been implicated as two distinct mechanisms underlying the activity of steroid receptors. The state of histone acetylation is positively mediated through complexes of histone acetyltransferases which are recruited through the ligand-dependent interaction of SRC-1 family members with the AF-2 activation domain and is negatively regulated by the interaction of complexes which contain histone deactylases and associate with receptors in the absence of ligand. The chromatin remodeling activity of BRG-1 containing complexes has also been shown to play an important role in nuclear receptor signaling. Studies using a yeast-based two hybrid system suggest a direct and hormone dependent association between BRG-1 and either ER or the retinoic acid receptor (RAR) in yeast (Ichinose et al., 1997). These findings may suggest an alternative mechanism of interaction between BRG-1 and ER that is either direct or relies upon an unrelated yeast protein capable of mediating an interaction between a liganded nuclear receptor and BRG-1. Other recent studies have suggested that these effects are more efficiently targeted to a stably integrated reporter than to one which is transiently transfected (Fryer and Archer, 1998). In these studies, we have shown that these two aspects of transcriptional activation are physically and functionally linked. SRC-1 serves not only as the link between an activated nuclear receptors and histone acetyltransferases, but also as the link between ligand binding and recruitment of a BRG-1 chromatin remodeling complex. Furthermore the ability of histone deactylase inhibitors to augment ER signaling depends on the function of BRG-1, suggesting that BRG-1 may act downstream of histone acetylation.

Previous studies provide a biochemical link between SRC-1 and the HAT-containing transcriptional coactivators CBP and p300 and suggest that this interaction contributes to nuclear receptor activation. Those studies coupled to our demonstration of a physical interaction between

SRC-1 and BRG-1 indicate that SRC-1 may represent a physical point of convergence between the contributions of CBP/p300-mediated coactivation and BRG-1-mediated nucleosome remodeling. This would suggest a model in which SRC-1 acts as a dynamic platform upon which different classes of coactivators can be recruited to an activated receptor. SRC-1 may be able to physically couple the contributions of these mechanisms to the regulation of ER function. Presently it is unclear whether these two mechanisms can be recruited to an activated receptor simultaneously, however several recent findings suggest that the functional coupling of histone acetylation and nucleosome remodeling may represent a general theme in transcriptional activation. Studies in yeast have shown phenotypic similarities between strains which carry mutations in members of a HAT complex and strains which carry mutations in Swi/Snf components. Significantly both phenotypes were rescued by mutations in the non-histone chromatin component, Sin1 (Pollard and Peterson, 1997). In another recent study, a protein which is structurally similar to CBP and p300 and therefore may contain an intrinsic HAT has been shown to be a component of the Swi/Snf complex (Dallas et al., 1998). Our data demonstrate that SRC-1 and BRG-1 exist in a complex and that this association accounts for the recruitment of BRG-1 to liganded ER. Furthermore, we demonstrate that BRG-1 is required for ER activation, and that its activity potentiates the positive effects of histone acetylation. Since it has been demonstrated that hyperacetylation of histones in core nucleosomes results in reduced affinity for DNA, (Paranjape et al., 1994) it is intriguing to consider a model, in which a hyperacetylated nucleosome is a more efficient substrate for the actions of BRG-1.

Methods

Production of monoclonal antibodies : A GST-SRC-1 fusion protein that represented amino acids 381 to 1440 of accession number HSU59302_1 was produced in E.Coli strain BL-21, pruified by conventional methodology and injected into mice in a three dose regimen. Following hybridoma production, individual colonies were screened for their ability to immunoprecipitate in vitro translated radiolabelled hSRC-1. Positive clones were subcloned and retested for a total of four rounds.

Cell lines, and metabolic labelling : MDA-MB-231, MCF-7 and SW-13 cells were grown in standard Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum, L-glutamine and antibiotics. All cultures were incubated at 37° C at 5% CO_2. For metabolic labelling, cells were washed thoroughly with 37° PBS and starved in serum-free and methionine-free DME for 30 to 60 minutes. Following starvation, 1mCi of ^{35}S-translabelling mixture (New England Nuclear) was added to the media, and cells were incubated for 3 hours under standard incubation conditions.

Whole Cell Extracts : Cells were harvested either directly from the culture dishes, or by trypsinization. Following thorough washing of cells in PBS, a lysis buffer NET-N {20mM Tris-Cl (pH 7.8), 10mM EDTA, 150mM NaCl, and 0.5% NP-40} and supplemented with 0.2mM PMSF, 10 µg/ml leupeptin and 10 µg/ml aprotinin was added directly to the plates or to a cell pellet. Cell suspensions were incubated 30 to 60 minutes at 4° C and centrifuged to remove debris. Lysates were supplemented with glycerol to a final concentration of 20% and stored at -80° C.

Immunoprecipitation reactions: Precipitation reactions were set up with approximately 0.5 ug of antibodies and whole cell extracts ranging in quantity from 100 µg to 2 mg. The antibody and extract were incubated in NET {20mM Tris-Cl (pH 7.8), 10mM EDTA, 150mM NaCl} for 30 minutes on ice. Following the incubation, 40 µl of a 25% (v/v) slurry of protein A sepharose beads (Pharmacia) was added, and the resulting suspension was incubated at 4° C with constant rotation. Suspensions were briefly centrifuged and aspirated. Pellets were resuspened in NET-N and washed a total of three times. Retained fractions were boiled in 2 X SDS sample dye,

resolved on 7.5% SDS-PAGE . Following electrophoresis, samples were either flourographed, subjected to western blot analysis by conventional methods.

Immunoprecipitation/re-immunprecipitation: Following a primary immune precipitation of metabolically labelled whole cell extracts (as described above), retained fractions were boiled in 50mM Tris-Cl (pH 7.5) 1% (v/v) SDS and 5mM DTT, and separated from protein A sepharose beads by centrifugation. Samples were then diluted 30 fold in NET and precipitated with 0.5 μg of a secondary antibody essentially as described above. Retained fractions were boiled in 2 X SDS sample dye resolved on 7.5% SDS PAGE and flourographed.

GST-pulldown assays: Glutathione-S-transferase fusion proteins, GST-ER-HBD and GST-ER–Δ534 were produced in E.Coli strain BL-21 as described. Fusion proteins were bound to GSH-agarose and used as affinity matrices, in the presence or absence of 1μM 17-β-estradiol, to enrich for interacting factors from 1 mg of whole cell extract. Retained fractions were vigorously washed in NET-N subjected to reprecipitation analysis as described above. In other experiments *in vitro* translated radiolabelled BRG-1 -/+ *in vitro* translated SRC-1 were subsituted for whole cell extract. Retained fractions were washed in NET-N resolved on 7.5% SDS PAGE and flourographed.

Transient transfections: SW-13 were plated at 1.5 X 10^5 per well in 6 well culture dishes in phenol-free DME supplemented with 10% dextran-coated charcoal-stripped fetal calf serum, L-glutamine and antibiotics. Transfections were carried out by the calcium phosphate method 12 to 20 hours after plating. At 12 hours post transfection, transfectants were removed and washed with

37° C PBS before being treated with media that was supplemented with ethanol (vehicle), 10nM estrogen or 250 nM Trichostatin A (TSA). Co-transfected as an internal control, into each point was 25-50 ng of a plasmid that contained the β-galactosidase coding sequence under the control of the the minimal promoter from HSV thymidine kinase. Experiments were done in triplicate and data are represented as the fold of induction of the ratio of luciferase to galactosidase activity. Error bars represent the SEM.

Acknowledgments

The authors wish to gratefully acknowledge Dr. J. Don Chen for the gift of a RAC3 expression plasmid, and also Dr. Ramesh Shivdasani, Dr. Mitch Lazar and Dr. Bettina Hanstein for helpful discussions. This work was supported by a National Institutes of Health grant # CA 57374-03 to M.B. and a U.S. Department of Defense Grant # DAMD 17-97-1-7069 to J.D.R.

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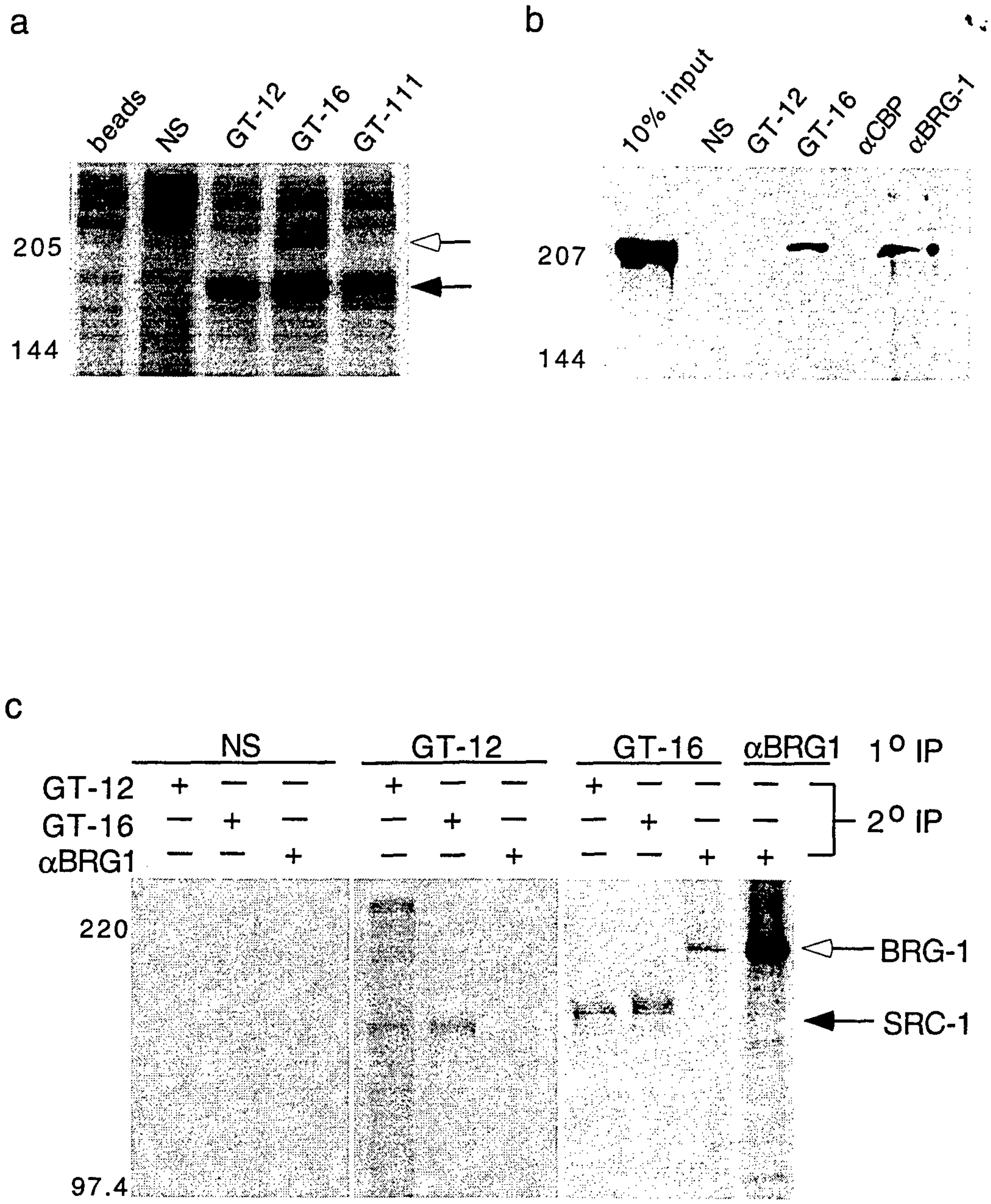
Figure 1 :*Monoclonal antibody GT-16 co-immunoprecipitates BRG-1 along with SRC-1.* **a:** The human breast carcinoma cell line MDA-MB-231 was metabolically labelled with ^{35}S-labelled methione and cysteine. These cells were lysed in NET-N supplemented with 0.2 mM PMSF, 10 µg/ml Leupeptin and 10 µg/ml Aprotinin and centrifuged to remove cellular debris. Lysates representing 1.5 X 10^6 cells were incubated with either a non-specific antibody (NS) or the mouse anti-human SRC-1 monoclonal antibodies GT-12, GT-16 and GT-111. Immune complexes were captured on protein A-sepharose beads, washed vigourously in NET-N, resolved on 7.5% SDS=PAGE and flourographed. The shaded arrow indicates a 160 kDa band that is precipitated by all three anti-SRC-1 antibodies, and appears by western blot analysis to be SRC-1. The unshaded arrow indicates a 200 kDa protein in the GT-16 immunoprecipitate that, by western blot analysis, is not recognized by anti-SRC-1 antibodies. Similar results were obtained using several other cell lines. **b:** Western blot analysis of anti-SRC-1 immunoprecipitates using a rabbit antiserum directed against human BRG-1 identifies the ~200 kDa factor in the GT-16 immunoprecipitate as BRG-1. Immunoprecipitation reactions were carried out essentially as in panel a. Following electrophoresis, resolved proteins were transferred to nitrocellulose and western blotted by standard methodology. **c:** Antibodies directed against BRG-1 re-precipitate the 200 kDa band that is co-immunoprecipitated by GT-16. MCF-7 cells were metabolically labelled, lysed and subjected to immunoprecipitation (as in panel **a**). Following the wash steps, retained fractions were eluted by boiling in a buffer containing 1% SDS and 5mM DTT. Eluates were diluted in NET buffer and corresponding secondary antibodies were added for immunoprecipitation. Retained fractions from the secondary immunoprecipitation were eluted by boiling in SDS sample dye, resolved on a 7.5% SDS PAGE and flourographed.

Figure 2: *The SRC-1/BRG-1 complex associates with the hormone binding domain of ER in a manner that is hormone and AF-2 dependent.* **a.** MCF-7 cells were metabolically labelled and lysed as described. Glutathione-S-Transferase fusion proteins representing the hormone binding domain of ER and an AF-2 deletion mutant (Δ-534) were immobilized on glutathione-linked sepharose and incubated with ethanol (vehicle), 10 nM 17-β-estradiol or 10 nM 4-hydroxy tamoxifen. These proteins were used as affinity matrices to enrich for factors that associate with ER. Following this enrichment, retained fractions were extensively washed, and subjected to elution and re-immunoprecipitation (as described in Figure 1C). The products of this immunoprecipitation were washed and eluted in SDS sample dye. The eluates were resolved on 7.5% SDS PAGE and flourographed. **b.** Similar GST-pulldown assays were performed using in vitro translated ^{35}S-labelled BRG-1and in vitro translated SRC-1. Following the pulldown, retained fractions were washed, resolved on a 7.5% SDS PAGE, and autoradiographed.

Figure 3: *BRG-1 coactivates estrogen signaling in a manner that is hormone and AF-2 dependent and is is enahnced by overexpression of SRC-1.* The adrenal carcinoma cell line SW-13 was transiently transfected with an estrogen reponsive luciferase reporter gene (ERE_2-tk-luc), expression plasmids encoding full length ER and ER Δ534 (pcDNA 3.1-hER and pcDNA 3.1-hER Δ534 respectively) an expression plasmid encoding hBRG-1 (pBJ-5-hBRG-1), and expression plasmid that encodes mSRC-1 (pcDNA 3.1-mSRC-1) and a β-galactosidase reporter for internal control of transfection efficency (tk-lacZ). Transfections were carriedout using the $CaPO_4$ precipitation method. Prior to transfection cells were plated at 1.5 X 10^5 cells per well in 6 well dishes and allowed to attach for 16 hours. Cells were washed in PBS and refed phenol-free DME supplemented with 10% dextran-coated charcoal stripped fetal calf serum. Cells were transfected with 4 μg of DNA per well for 16 hours, washed twice in 37° C PBS and refed in the same media supplemented with either ethanol (vehicle) or 10nM 17-β-estradiol. Cells were harvested at 20 hours post feeding and assayed for luciferase and β--galactosidase activity by conventional methods. Experiments were done in triplicate and data are expressed as the fold induction of the ratio of luciferase activity to β-galactosidase activity. Error bars represent the SEM.

Figure 4: *Overexpression of CBP or inhibition of histone deacetylation enhance BRG-1 mediated coactivation of Estrogen signaling.* **a:** Transient transfection of SW-13 cells was perofrmend as described. In these studies an plasmid encoding the transcriptional coactivator CBP (pcDNA-CBP) was co-transfected along with the other factors indicated. **b.** Transient transfections were done as described with the additional treatment of the cells with Trichostatin A (TSA). Cells were treated at 16 hours post transfection with either ethanol (vehicle) 10 nM 17-β-estradiol (E2), 250 nM TSA or E2 plus TSA. **c:** Transient transfections were done as described including a plasmid that encoded an ATPase deficient mutant of BRG-1 (pBJ-5 hBRG-1 K798R). Experiments were done in triplicate and data expressed as as the fold induction of the ratio of luciferase activity to β-galactosidase activity. Error bars represent the SEM.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beads
NS
GT-12
GT-16
GT-111
205
144
b
10% input
NS
GT-12
GT-16
αCBP
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207
144
c
NS
GT-12
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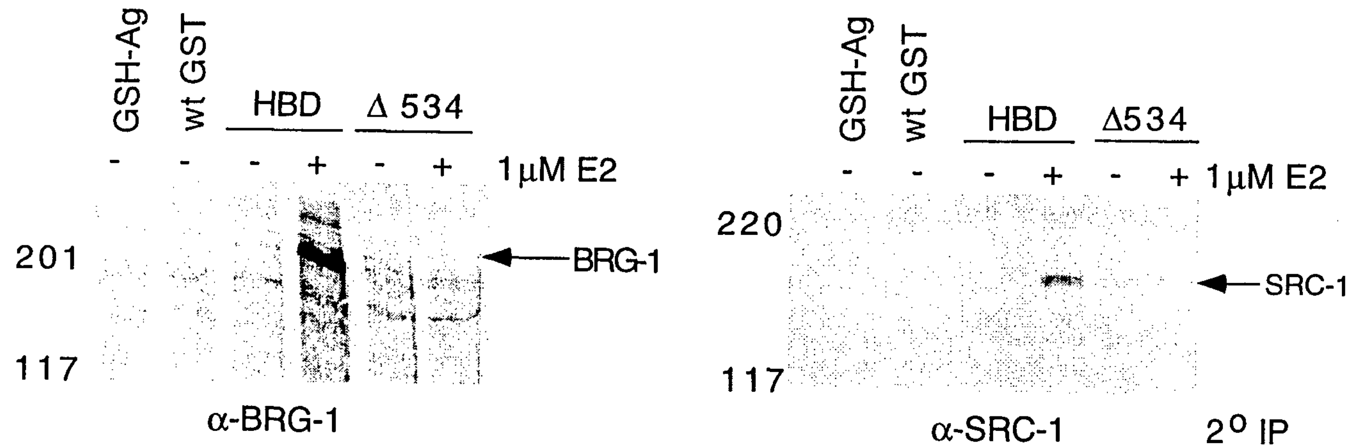
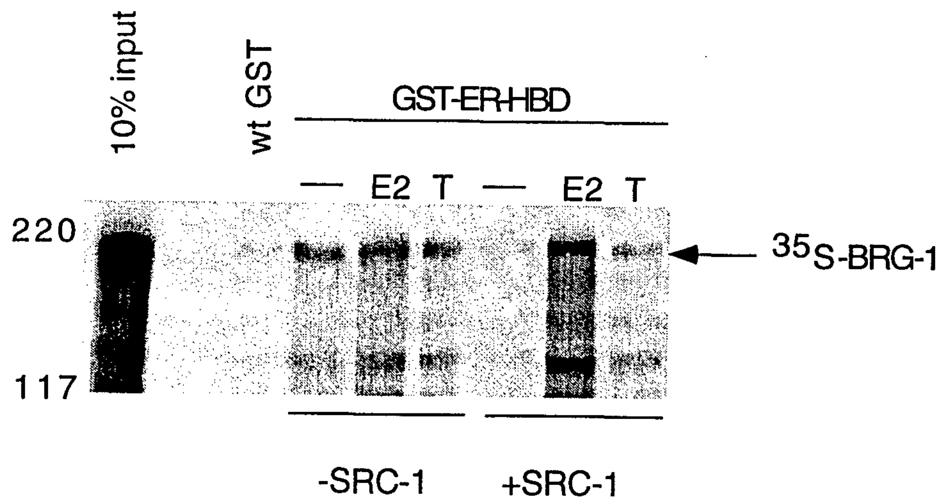
BRG-1
SRC-1

a



GSH-Ag
wt GST
HBD
Δ 534
- - - + - + 1 µM E2
201
117
BRG-1
α-BRG-1
GSH-Ag
wt GST
HBD
Δ534
- - - + - + 1 µM E2
220
117
SRC-1
α-SRC-1
2° IP

b



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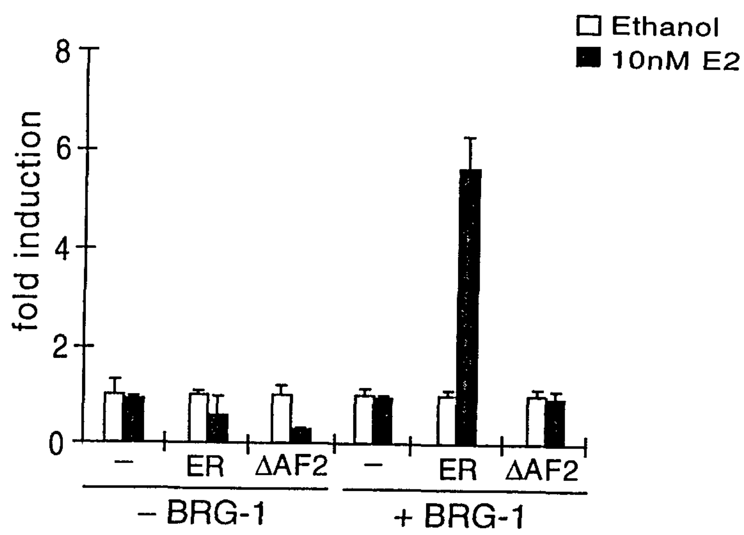
wt GST
GST-ER-HBD
— E2 T — E2 T
220
117
35S-BRG-1
-SRC-1
+SRC-1

a



Ethanol
10nM E2
fold induction
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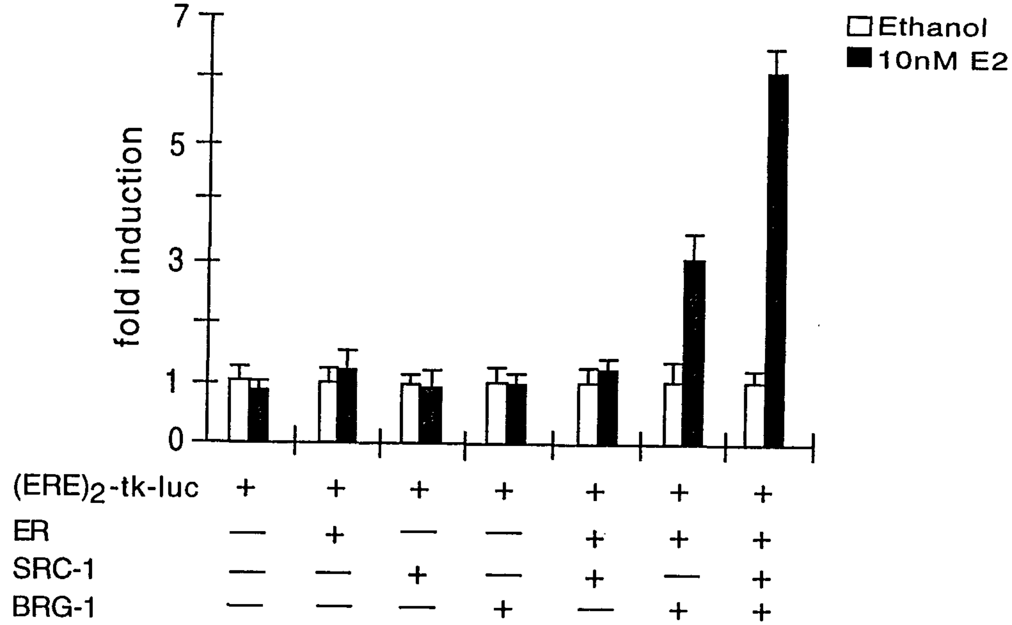
6
4
2
0
−
ER
ΔAF2
−
ER
ΔAF2
− BRG-1
+ BRG-1

b



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fold induction
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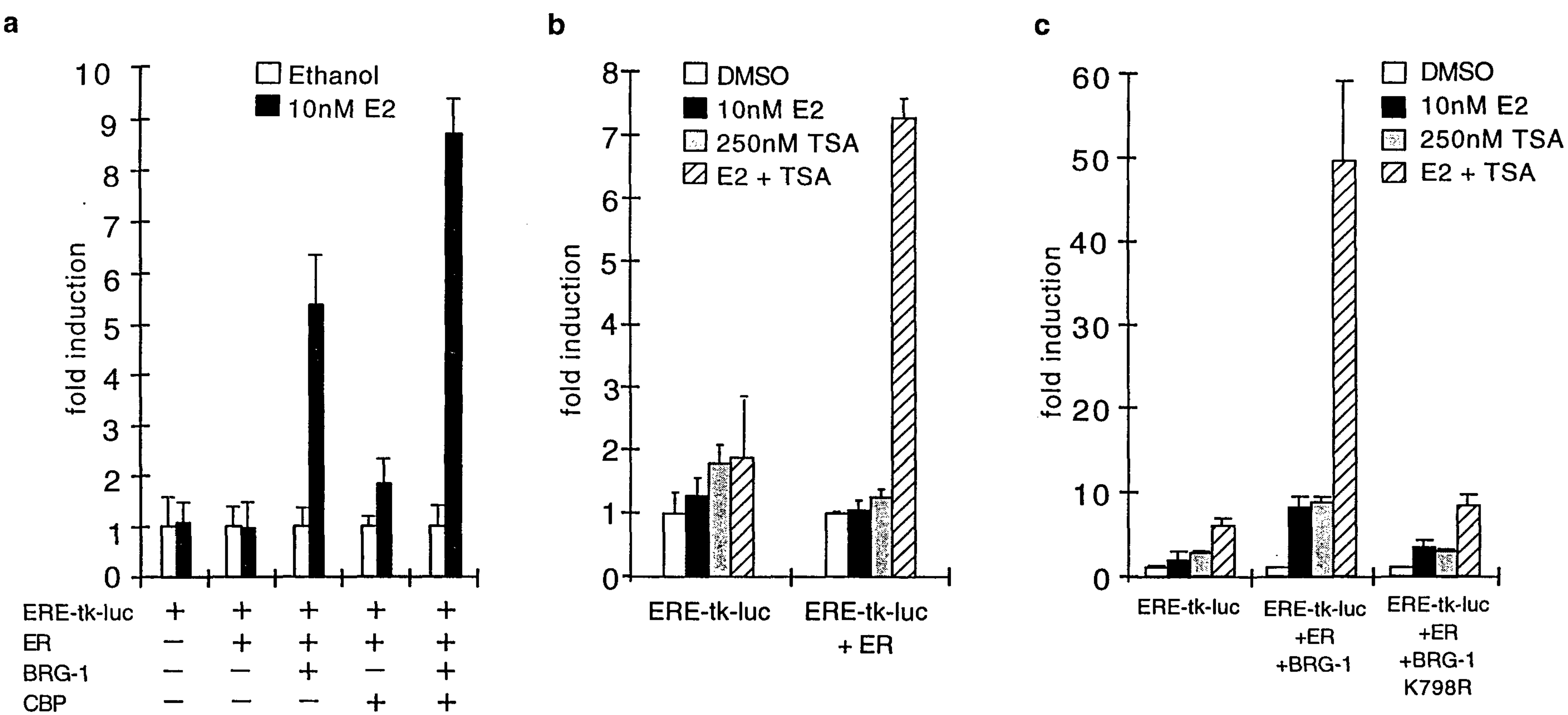
5
3
1
0
(ERE)2-tk-luc
ER
SRC-1
BRG-1



a
b
c
fold induction
Ethanol
10nM E2
DMSO
250nM TSA
E2 + TSA
ERE-tk-luc
ER
BRG-1
CBP
ERE-tk-luc + ER
ERE-tk-luc +ER +BRG-1
ERE-tk-luc +ER +BRG-1 K798R

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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Rec'd 8/16/2001

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 9 August 2001

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Encl